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Lipid Biomarkers and Carbon Isotope Signatures of a Microbial (*Beggiatoa*) Mat Associated with Gas Hydrates in the Gulf of Mexico

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White and orange mats are ubiquitous on surface sediments associated with gas hydrates and cold seeps in the Gulf of Mexico. The goal of this study was to determine the predominant pathways for carbon cycling within an orange mat in Green Canyon (GC) block GC 234 in the Gulf of Mexico. Our approach incorporated laser-scanning confocal microscopy, lipid biomarkers, stable carbon isotopes, and 16S rRNA gene sequencing. Confocal microscopy showed the predominance of filamentous microorganisms (4 to 5 μm in diameter) in the mat sample, which are characteristic of *Beggiatoa*. The phospholipid fatty acids extracted from the mat sample were dominated by 16:1ω7c/t (67%), 18:1ω7c (17%), and 16:0 (8%), which are consistent with lipid profiles of known sulfur-oxidizing bacteria, including *Beggiatoa*. These results are supported by the 16S rRNA gene analysis of the mat material, which yielded sequences that are all related to the vacuolated sulfur-oxidizing bacteria, including *Beggiatoa*, *Thioploca*, and *Thiomargarita*. The $\delta^{13}C$ value of total biomass was −28.6‰; those of individual fatty acids were −29.4 to −33.7‰. These values suggested heterotrophic growth of *Beggiatoa* on organic substrates that may have $\delta^{13}C$ values characteristic of crude oil or on their by-products from microbial degradation. This study demonstrated that integrating lipid biomarkers, stable isotopes, and molecular DNA could enhance our understanding of the metabolic functions of *Beggiatoa* mats in sulfide-rich marine sediments associated with gas hydrates in the Gulf of Mexico and other locations.

Microbial mats dominated by sulfur-oxidizing *Beggiatoa* and *Thioploca* spp. are widespread in estuarine, continental shelf, deep-sea hydrothermal vent, and cold-seep environments, where reduced sulfur species are abundant. Examples include the Bay of Concepción in Chile (10, 11, 22, 46, 56, 58), the Monterey Canyon of California (3, 34), the Guaymas Basin (18, 19, 20, 42), Tokyo Bay (23, 55), and cold seeps in the North Atlantic (47). Some of these organisms can reach biomass densities of tens to hundreds of grams (wet weight) per square meter in surface sediments (10, 56, 57).

Beggiatoa and *Thioploca* can grow autotrophically, heterotrophically, and facultatively or mixotrophically (13, 16, 24, 39, 41, 61). These organisms also deposit internal globules of elemental sulfur formed by oxidation of reduced sulfur compounds (16). Furthermore, some *Beggiaota* or *Thioploca* species accumulate high concentrations of nitrate in their vacuoles and can use the nitrate as an electron acceptor for oxidation of reduced sulfur compounds under anaerobic conditions (3, 10, 34). Clearly, these organisms play important roles in the cycling of carbon, sulfur, and nitrogen in the marine environments.

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Beggiatoa mats occur widely in association with surface-breaching gas hydrates and related chemosynthetic communities in the Gulf of Mexico (6, 26, 28, 29, 49, 50, 52). Despite the ecological importance of *Beggiatoa*, the predominant pathways for carbon cycling within the *Beggiatoa* mats are not well defined. Here, we addressed this deficiency through an integrated study employing laser-scanning confocal microscopy (LSCM), lipid biomarkers, stable carbon isotopes, and 16S rRNA gene sequencing. Our results are consistent with enzyme assays of *Beggiaota* mats in the Gulf of Mexico, which demonstrate the presence of heterotrophic metabolism and the lack of autotrophic metabolism (43, 45). Our study also provides a biogeochemical perspective on ecological functions of *Beggiaota* mats in sulfide-rich environments.

MATERIALS AND METHODS

Sample collection. During a 2002 cruise of the R/V *Seaward Johnson II* in the Gulf of Mexico, massive bacterial mats (*Beggiatoa*) were observed covering the sediment surface over a gas hydrate mound at Green Canyon (GC) leasing block GC 234, which has a water depth of about 540 m (Fig. 1). A suction device equipped on the Johnson Sea-Link submersible was used to collect orange mat from the surface of the mound. Care was taken so that the device would collect only the mat material, with little or no contamination from underlying sediment. Upon return to the ship deck, the filamentous mat material was immediately removed from the Plexiglas sample chamber and stored in a −20°C freezer for approximately 4 days before being transferred to a −80°C freezer in our home laboratory. One portion of the frozen mat was lyophilized (~0.4 g [dry weight]) for lipid extraction and determination of carbon isotopes of bulk organic matter.

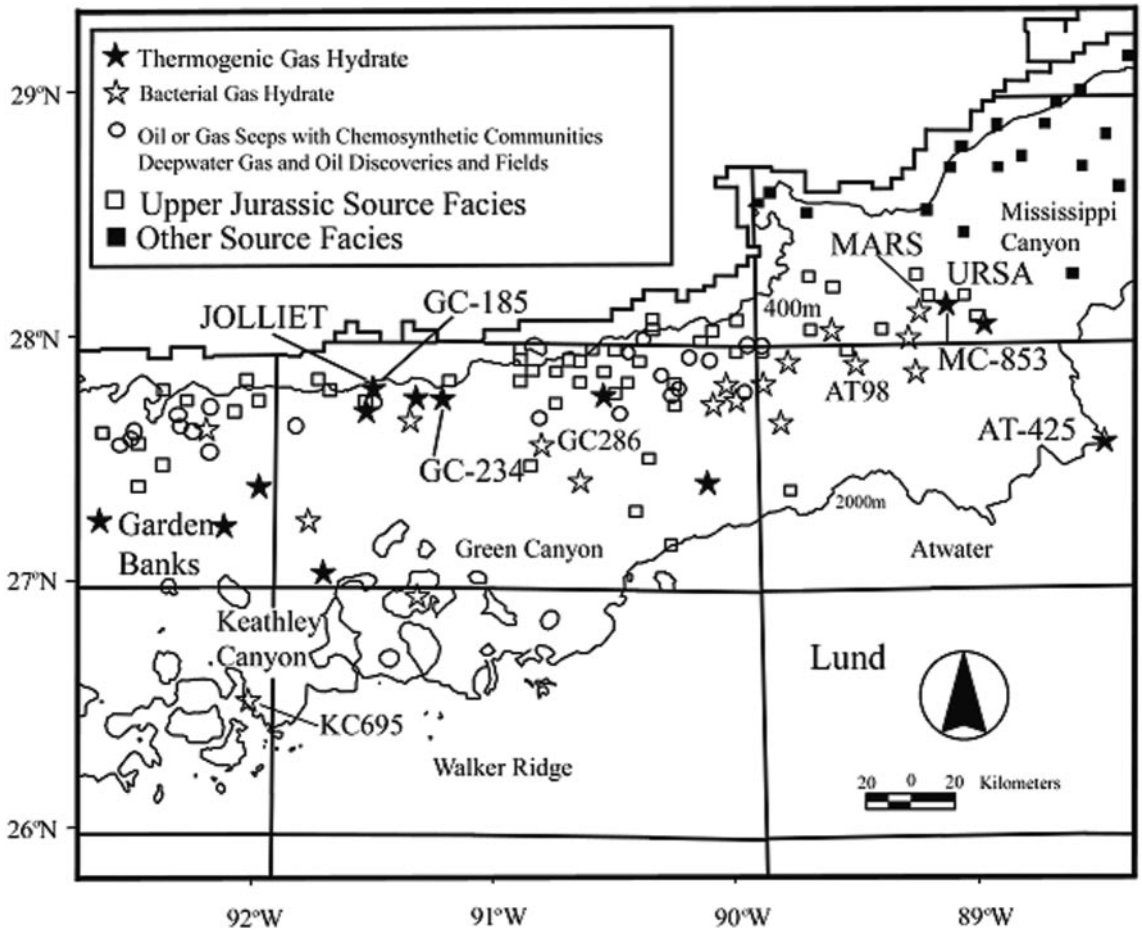

FIG. 1. Location of the Green Canyon region in the Gulf of Mexico. The *Beggiatoa* mat for this study was collected at GC 234 during dive 4438 in 2002. This figure was modified from reference 53.

Another portion (~0.5 g [wet weight]) was kept at −80°C for molecular DNA analysis.

LSCM. An LSCM system was used to observe the morphology and cell structure of *Beggiatoa*, using previously described methods (4). An aliquot (10 μl) of mat slurries made from the mat material and distilled water was pipetted onto slides and dried for 10 min at 65°C. A 2% hydrolyzed gelatin solution in 10 μl of phosphate-buffered saline (pH 7) was then layered over the heat-fixed sample and allowed to dry for 10 min at 65°C. The prepared sample was then stained with 10 μl of a 10-μg/ml 4,6-diamino-2-phenylindole (DAPI) (Sigma) solution for 10 min and rinsed with distilled water. The slides were mounted with a drop of SlowFade (Molecular Probes Inc., Eugene, Oreg.) and examined with a 510 LSCM (Carl Zeiss, Inc., Thornwood, N.Y.). Methanotrophic bacteria were observed through the application of specific fluorescent antibodies (5).

Lipid extraction. The lyophilized mat material was used for lipid extraction according to the procedure of White et al. (68). This procedure employed a single-phase organic solvent system comprised of chloroform, methanol, and aqueous 50 mM phosphate buffer (pH 7.4) in a ratio of 1:2:0.8 (vol/vol/vol). After overnight extraction, chloroform and nanopure water were added to the extract in equal volumes, which resulted in a two-phase system. The lipids confined to the lower phase were collected and fractionated on a silicic acid column into neutral lipids, glycolipids, and polar lipids (12). The polar lipids were treated by mild alkaline methanolysis to produce fatty acid methyl esters (FAMEs).

The FAMEs were identified by using an Agilent 6890 series gas chromatograph (GC) interfaced with an Agilent 5973 mass selective detector. The GC was equipped with a 60-m nonpolar column (0.25-mm internal diameter, 0.25-μm film thickness). The injector temperature was maintained at 230°C, and the detector temperature was 300°C. The column temperature was programmed at 60°C for 1 min, ramped at 20°C/min to 150°C, and held for 4 min. This was followed by ramping at 7°C/min to 230°C and holding for 2 min and finally by ramping at 10°C/min to 300°C and holding for 3 min.

Mass spectra were determined by electron impact at 70 eV. Methyl heneicosanoate was used as the internal standard. The FAMEs were expressed as equivalent peaks against the internal standard. Double-bond positions of monounsaturated FAMEs were determined by GC-mass spectrometry (GC-MS) analysis of the dimethyl disulfide adducts (44). *cis* and *trans* isomers of compounds were identified based on known standards.

Stable carbon isotopes. Carbon isotope compositions of the FAMEs were determined in duplicate as described by Zhang et al. (70), using an HP 6890 gas chromatograph connected to a Finnigan MAT Delta Plus-XL mass spectrometer. Each measurement was corrected for the methyl moiety (69, 70, 72). The mean and standard deviation of the duplicate measurements were reported for individual fatty acids.

Carbon isotope compositions of total biomass were determined with bulk samples after acidification in 10% HCl. The $^{13}C/^{12}C$ ratio of the total biomass was then determined on a Delta Plus isotope ratio mass spectrometer with a precision of ±0.2‰.

16S rRNA gene analysis. The frozen aliquot (0.5 g) of the mat material was extracted for total DNA by using a commercial DNA extraction kit (MO Bio Lab Inc., Solana Beach, Calif.). Eubacterium-specific primers sets 27F-1492R and 357F-517R (8) were used for the nested PCR amplification of the DNA sample. The final PCR products (~200 bp) were analyzed by denaturing gradient gel electrophoresis (DGGE) (37). The identified DNA bands were excised and extracted with a kit from Qiagen (Valencia, Calif.) and sequenced by using BigDye version 3.1 chemistry (Applied Biosystems, Foster City, Calif.) and an ABI 377 DNA sequencer. Sequences were aligned in ClustalX (version 1.8), and phylogenetic reconstructions were performed in PHYLIP (version 3.6e).

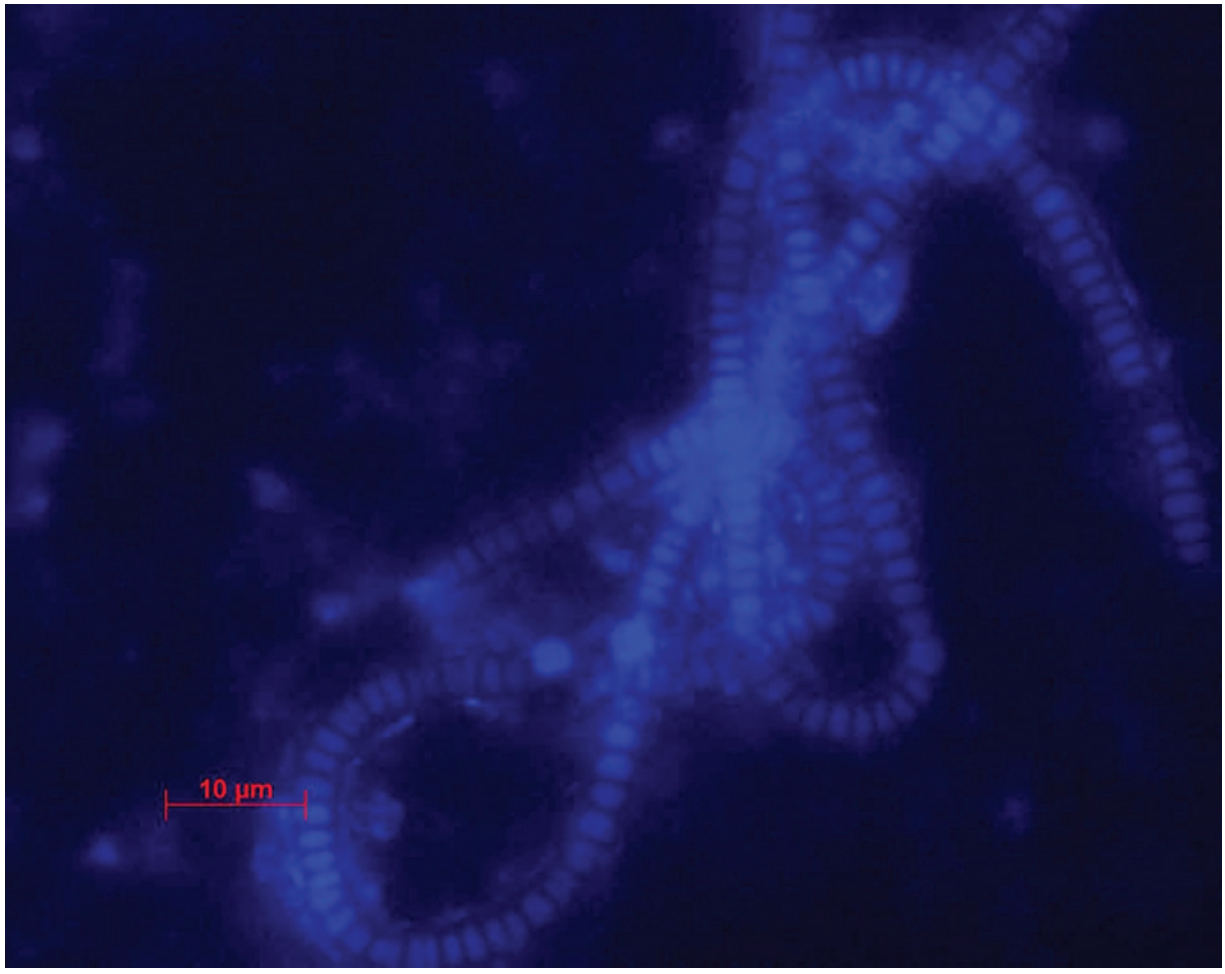

FIG. 2. DAPI-stained filaments characteristic of *Beggiatoa*-like microorganisms. The bright compartments are likely vacuoles, which may contain nitrate for anaerobic oxidation of hydrogen sulfide.

RESULTS AND DISCUSSION

LSCM. Observation under LSCM demonstrated the predominance of filamentous microorganisms (Fig. 2). The filaments were 4 to 5 μm in width and were characterized by bright central compartments (Fig. 2). The morphology of these filaments was consistent with the description of vacuolated *Beggiatoa* or *Thioploca*, which use the vacuoles for storage of nitrate (31, 42).

Microbial mats collected during a previous study in the Gulf of Mexico contained nonpigmented *Beggiatoa* filaments ranging in diameter from <25 to >85 μm and pigmented filaments ranging from <20 to 65 μm (45). Both types of filaments were dominated by diameters of 25 to 45 μm; however, filament diameters of <25 μm were more abundant in pigmented samples than in nonpigmented samples (45). The diameters of *Beggiatoa* organisms described in this study may be at the lower end of the range for pigmented filaments observed by Nikolaus et al. (45).

The size of filaments may reflect changing environments occupied by the sulfur-oxidizing bacteria. For example, Mussmann et al. (36) observed that narrow *Beggiatoa* species were present in upper sediment layers, whereas wide *Beggiatoa* species were predominant in deeper sediment layers. The larger filaments with a larger associated volume of stored nitrate may enable those species to stay longer in deeper anoxic sediments, where the nitrate serves as an alternative electron acceptor for anaerobic oxidation of sulfide (36). Jørgensen (21) also observed that *Beggiatoa* spp. of small sizes (3 to 5 μm) were relatively more abundant at the surface than in deeper sediments.

PLFA. Phospholipid fatty acids (PLFA) in the mat sample were dominated by 16:1ω7c (53.6%), 16:1ω7t (12.8%), 16:0 (8.3%), and 18:1ω7c (16.6%) (Table 1). *iso*- and *anteiso*-fatty acids (i.e., i15:0, a15:0, i17:0, and a17:0), which are characteristic of sulfate-reducing bacteria (27, 63, 67, 69), were minor components (less than 1% each) of the total PLFA (Table 1).

The fatty acid pattern reported here is consistent with fatty acid profiles of *Beggiatoa*, *Thioploca*, and other sulfur-oxidizing bacteria (14, 15, 17, 33). For example, McCaffrey et al. (33) performed fatty acid analysis of two *Thioploca* species from the Peru upwelling region. In these species, 16:1ω7c accounted for 40.3 to 42.5%, 18:1ω7c accounted for 36.0 to 37.8%, and 16:0 accounted for 17.3 to 18.0% (33). Jacq et al. (17) analyzed the lipid profiles of a whitish mat from a subtidal hydrothermal

TABLE 1. PLFA of the orange *Beggiatoa* mat at GC 234 (dive 4438)

PLFA	mol%
a12:0	0.04
12:0	0.44
i13:0	0.11
a13:0	0.05
i14:0	0.12
14:1	0.31
14:1ω5c	0.08
14:0	1.28
br14:1	0.04
i15:0	0.72
a15:0	0.73
15:1	0.07
15:0	0.35
br15:0	0.07
i16:0	0.05
a16:0	0.18
16:1ω7c	53.61
16:1ω7t	12.78
16:1	0.96
16:0	8.32
br16:0	0.03
br16:1	0.09
10Me16:0	0.21
11Me16:0	0.04
i17:0	0.10
a17:0	0.19
cy17:0	0.19
17:0	0.13
br17:1	0.03
18:2	0.18
18:2ω6	0.08
18:1ω9c	0.34
18:1ω7c	16.64
18:1	0.12
18:0	0.32
19:1	0.03
cy19:0	0.04
19:0	0.04
2Me19:0	0.09
20:4ω6	0.15
20:5ω3	0.32
20:1ω9c	0.09
20:1	0.05
20:0	0.03
22:6	0.13
Total	100.0

vent in southern California, which contained "*Thiothrix*-like" bacteria. Fatty acids of these bacteria were dominated by 16:0, 16:1ω7c, 18:0, and 18:1ω7c, which were similar to those determined for a *Beggiatoa* sample collected from a spring in Newport, Fla. (17). Dominance of 16:1ω7c and 18:1ω7c has also been observed in thiotrophic bacterial mats in the Barbados Trench (14) and in the deep-sea hydrothermal vents on the Mid-Atlantic Ridge (15). These results suggest that 16:1ω7 and 18:1ω7 can be used as signature biomarkers for sulfur-oxidizing bacteria in H_2S-rich marine sediments.

Lipid biomarkers such as 16:1ω6 and 16:1ω8 are commonly found in type I methanotrophs, whereas biomarkers such as 18:1ω6 and 18:1ω8 are commonly found in type II methanotrophs (32, 66). None of these biomarkers were detected in the mat sample (Table 1), suggesting an extremely low abundance of methanotrophs in the mat community. This conclusion is consistent with our microscopic observation of the scarcity of methanotroph cells. Furthermore, archaeol, a diphytanyl glycerol diether that is common in archaea and in sediments associated with anaerobic oxidation of methane, was found in very low abundance (data not shown). This result is consistent with the low abundance of PCR products of *Crenarchaeota* (see "16S rRNA gene sequences" below).

Carbon isotopes. The total biomass of the *Beggiatoa* mat had a $\delta^{13}C$ value of −28.6‰, which was similar to the $\delta^{13}C$ of *Beggiatoa* mats (−26.6 to −27.9‰) at other gas hydrate locations in the Gulf of Mexico (2, 26, 52). Isotopic compositions of PLFA could be determined only for 16:0, 16:1ω7c, 16:1ω7t, and 18:1ω7c, which occurred at high enough concentrations for measurements with the GC-isotope ratio MS (GC-IRMS). The $\delta^{13}C$ values of these fatty acids were −29.4‰ ± 0.3‰ for 16:0 ($n = 2$ for this and the following fatty acids), −32.2‰ ± 0.6‰ for 16:1ω7c, −36.7‰ ± 0.0‰ for 16:1ω7t, and −33.7‰ ± 0.1‰ for 18:1ω7c. Because part of the 16:1ω7c peak coeluted with that of 16:1ω7t on GC-IRMS, measurements of their isotopic compositions were likely compromised. A composite $\delta^{13}C$ value was thus obtained by integrating the $\delta^{13}C$ values of these two isomers, using the weight percentage of mass 44 [V] of each peak (72). The integrated $\delta^{13}C$ value was −33.7‰ ± 0.6‰ for 16:1ω7c/t. The carbon isotope compositions of biomass and lipid biomarkers in this study clearly indicated that *Beggiatoa* did not use methane as the carbon substrate (the $\delta^{13}C$ was <-50‰ for the local methane source [54]).

There are several possible pathways for carbon metabolism by *Beggiatoa* and similar species. One pathway may involve direct oxidation of nonmethane hydrocarbons or use their organic by-products for heterotrophic growth (45). *Beggiatoa* spp. have been shown to grow on volatile organic acids such as acetate, lactate, or pyruvate (13, 16, 38, 41, 61). Direct oxidation of hydrocarbons by *Beggiatoa*, however, has not been demonstrated in culture studies. Another possible pathway is chemoautotrophic growth of *Beggiatoa* by fixation of CO_2 using the Calvin cycle (39, 40). *Beggiatoa* can also grow facultatively or mixotrophically by using inorganic and organic compounds as the energy sources (13, 16, 42, 45, 60, 61).

In this study, estimates of isotopic fractionations between biomass and CO_2 suggested that it was unlikely that *Beggiatoa* used the Calvin cycle for autotrophic growth. First, Aharon et al. (1) determined $\delta^{13}C$ values of −26.0 to −27.8‰ for dissolved inorganic carbon in the top sediment layer above hydrocarbon seeps in the Gulf of Mexico. Because a fractionation of ~−10.7‰ exists between dissolved CO_2 and dissolved inorganic carbon (dominated by bicarbonate) at the in situ temperature (~10°C) (35), the actual isotope compositions of the respired CO_2 can be as low as −38.2‰. Second, if *Beggiatoa* used this light CO_2 for chemoautotrophic growth, the biomass should have had a $\delta^{13}C$ value close to or below −60‰, given the typical fractionations of 20 to 26‰ between biomass and CO_2 for autotrophs using the Calvin cycle (30, 48, 51, 59). Our measured $\delta^{13}C$ value of biomass (−28.6‰) was significantly higher. On the other hand, our measured value was consistent with heterotrophic growth on soluble organic substrates derived from degradation of hydrocarbons that had isotopic compositions ranging from −27.0 to −34.0‰ (69, 71). The results were also consistent with enzyme assays of the orange mat

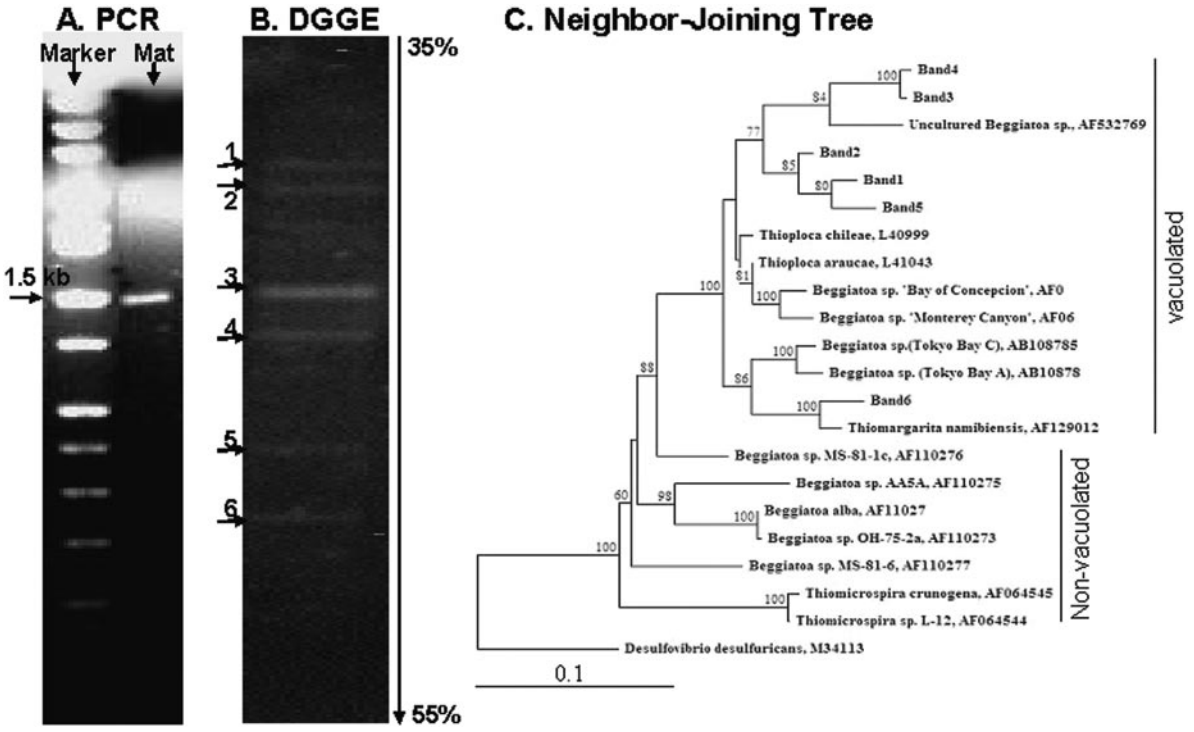

FIG. 3. PCR product (A), DGGE (B), and neighbor-joining tree (C) of a *Beggiatoa* mat at GC 234. The scale bar in panel C indicates 10 substitutions per 100 nucleotide positions. The sulfate-reducing bacterium *Desulfovibrio desulfuricans* was used as the root of the phylogenetic tree.

from the Gulf of Mexico, which showed great heterotrophic activity but very low RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) activity (43, 45). Nelson and McHatton (43) also concluded that *Beggiatoa* mats in the Gulf of Mexico are typically less autotrophic and more heterotrophic than any natural *Beggiatoa* mats previously studied.

Mixotrophic growth can incorporate some CO_2 during biosynthesis (16). However, the insignificant RuBisCO activity associated with the orange mat suggests that CO_2 fixation should be a minor pathway for cellular production by the mixotrophic *Beggiatoa*.

16S rRNA gene sequences. 16S rRNA gene sequencing provided phylogenetic evidence supporting the predominance of *Beggiatoa*-type species in the mat material. The PCR product amplified from environmental nucleic acids showed a clear band at 1.5 kb (Fig. 3A), which identified the bacterial DNA. DGGE of the PCR product showed six discrete bands (Fig. 3B), which were determined to represent six different sequences (Fig. 3C).

All six identified sequences are within the family *Beggiatoaceae* of the gamma-proteobacteria (25, 62, 64, 65). Furthermore, all sequences are comparable to those of species of vacuolated *Beggiatoa*, *Thioploca*, or *Thiomargarita*. For example, bands 3 and 4 had 98.7% similarity with an uncultured *Beggiatoa* sp.; bands 1, 2, and 5 had 97.1 to 97.5% similarity with *Thioploca chileae*; and band 6 had 99.9% similarity with *Thiomargarita namibiensis* (Fig. 3C).

Previous studies of *Beggiatoa* in the Gulf of Mexico have reported finding only the vacuolated species at a variety of depths below the sediment-water interface (43, 45). Our findings are consistent with those observations. The sizes of the vacuoles we observed, however, were small. This may be because we focused on *Beggiatoa* mats living at the water-sediment interface, where oxygen may be the dominant electron acceptor for oxidation of hydrogen sulfide. Consequently, the bacteria may not need large vacuoles for storing nitrate.

While other bacterial species were not detected by using the 16S rRNA gene approach, the PCR product did indicate the presence of *Crenarchaeota* (data not shown). However, the DNA abundance was too low to allow further analysis of the *Crenarchaeota* distribution in this mat sample.

Biogeochemical implications. *Beggiatoa* spp. play an important role in the biogeochemistry of surface sediment by coupling carbon cycling to oxidation of reduced sulfur. The bacteria also enhance anaerobic processes below the mat and/or the surface sediments by consuming oxygen (7). Furthermore, the biomass of *Beggiatoa* can add considerable amounts of bioavailable organic carbon to the underlying sediment, which can enhance microbial activities for nitrate reduction, iron reduction, and/or sulfate reduction.

Understanding the lipid profiles of *Beggiatoa* spp. and their isotopic signatures allows us to better evaluate their contributions in carbon cycling in marine sediments. For example, at the gas hydrate site at GC 286 (Fig. 1), where *Beggiatoa* mats are also abundant (R. Sassen, unpublished data), the $\delta^{13}C$ values of 16:1 and 18:1 phospholipid fatty acids ranged from -21.0 to $-33.1‰$, whereas in the same sample, the $\delta^{13}C$ values of *iso*- and *anteiso*-C_{15} and -C_{17} phospholipid fatty acids, which are characteristic of sulfate-reducing bacteria, were significantly lower (-57.1 to $-65.8‰$) (69). These results, in light of this study, indicate that *Beggiatoa* (with higher $\delta^{13}C$ values) may have contributed to the 16:1 and 18:1 lipid pool in

the underlying sediments, whereas sulfate-reducing bacteria that oxidize ^{13}C-depleted methane contribute to the pool of the branched fatty acids in the same sediment (69). Elvert et al. (9) also reported that in the *Beggiatoa*-covered sediment core associated with gas hydrate, 16:1ω7c, 16:1ω7t, and 18:1ω7c in the top 4 cm were significantly enriched in ^{13}C ($\delta^{13}C = -31$ to $-46‰$) relative to biomarkers (i.e., *iso*- and *anteiso*-15:0 and -17:0 and cy17:0) of the sulfate-reducing bacteria ($\delta^{13}C = -58$ to $-101‰$). Again, these results suggest that sediment biomass may be contributed by *Beggiatoa* growing on nonmethane substrate and by sulfate-reducing bacteria growing on methane-derived carbon. Because *Beggiatoa* normally live above the sulfate-reducing zone, the biomarkers of *Beggiatoa* in deeper sediments most likely represent deposition from top layers where they live. Thus, examining the distribution of lipid biomarkers and their isotopic compositions will allow us to better understand the biological sources contributing to the carbon pool in marine sediments.

Summary. The species diversity and ecological functions of *Beggiatoa* were studied in an orange mat in the Gulf of Mexico where gas hydrates and cold seeps occur. The application of laser-scanning confocal microscopy, lipid biomarkers, and 16S rRNA gene sequencing allowed us to link morphology and species identity to their phenotypic properties. Furthermore, isotopic compositions of lipid biomarkers allowed us to elucidate the carbon-cycling pathways of *Beggiatoa* and the potential carbon substrates for their metabolism. Specifically, confocal microscopy identified small vacuolated (~5-μm diameter) *Beggiatoa*-type filaments. The dominant lipid biomarkers (16:1ω7c/t and 18:1ω7c) were characteristic of sulfide-oxidizing bacteria, including *Beggiatoa*, which was supported by 16S rRNA gene sequencing. The isotopic compositions of total biomass and lipid biomarkers further implied that *Beggiatoa* in the mat grew heterotrophically using organic carbon principally derived from degradation of nonmethane hydrocarbons. These results demonstrate that integration of microscopy, lipid biomarker and stable isotope analysis, and molecular DNA analysis is an effective approach for understanding the community structure and ecological functions of microorganisms in natural environments.

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